

CHROMATOGRAPHY

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CHROMATOGRAPHY

Some 50 years ago a Russian botanist devised a way of separating the pigments of a green leaf. Now the method has been developed to fractionate a host of mixtures of subtly different substances

by William H. Stein and Stanford Moore

If a petroleum ether solution of chlorophyll is filtered through a column of an adsorbent (I use mainly calcium carbonate which is stamped firmly into a narrow glass tube), then the pigments, according to the adsorption sequence, are resolved from top to bottom into various colored zones. . . . Like light rays in the spectrum, so the different components of a pigment mixture are resolved on the calcium carbonate column according to a law and can be estimated on it qualitatively and quantitatively. Such a preparation I term a chromatogram, and the corresponding method, the chromatographic method. It is self-evident that the adsorption phenomena described are not restricted to the chlorophyll pigments, and one must assume that all kinds of colored and colorless chemical compounds are subject to the same laws.

IN THESE FEW words a 34-year-old Russian botanist named Michael Tswett in 1906 described a new technique which, to quote a later comment, "was destined to influence the life of man and beast the world over." As the author of this comment, H. H. Strain of the Carnegie Institution of Washington at Stanford University, went on to observe, Tswett's chromatographic technique provided scientists with a "particularly efficient procedure for the preparation of chemical compounds in a high state of purity. Isolation and identification of chemical substances, prerequisites to investigations of composition and molecular structure, were brought to a new state of perfection." Chromatography eventually made feasible the isolation of "numerous ephemeral substances" such as vitamins, drugs and pigments, and revealed "undreamed-of reactions" in living cells.

It would be pleasant to record that Michael Tswett received in his lifetime the acclaim warranted by his discovery. Such, however, is not the case. A quarter of a century elapsed before science began to make wide use of his findings. Since 1930, however, the applications

of chromatography have been legion. By now literally thousands of papers have appeared describing investigations in which the method has been used in one way or another. This article will attempt to trace in broad outline the development and application of the technique.

Chromatography utilizes the phenomenon of adsorption. This may be defined as the adhesion of a thin layer of molecules of a gas, a dissolved substance or a liquid to the surface of a solid body. Adsorption is well known as a method for removing impurities. For example, the common gas mask contains charcoal to adsorb the molecules of a noxious gas contaminating the air breathed in by the wearer. In the sugar industry charcoal is employed to remove colored impurities from concentrated cane-sugar solutions so that a clear white crystalline product may be obtained.

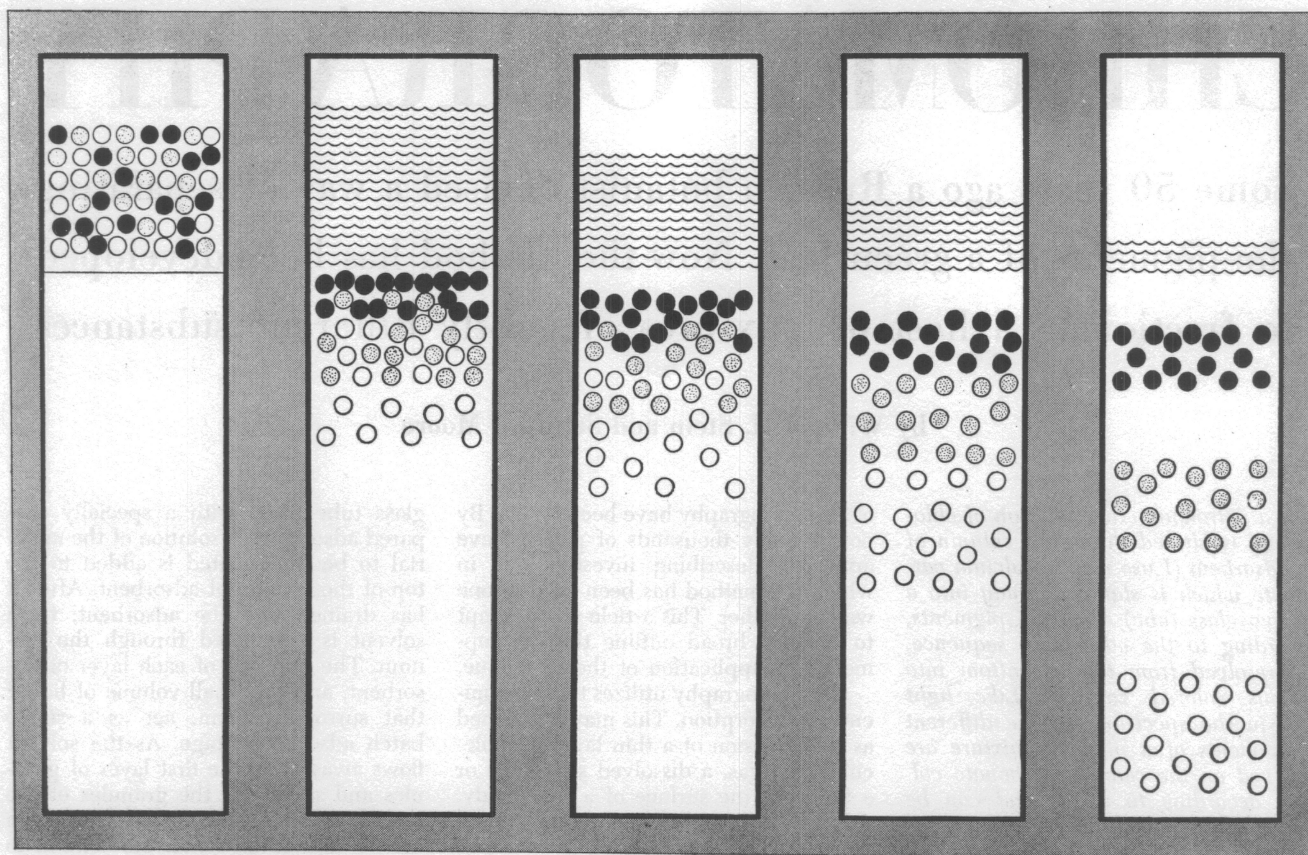
Adsorption is also frequently used for the concentration of a desired substance as, for example, in the isolation of the antibiotic drug streptomycin from its mold culture. The drug was adsorbed on charcoal, along with some other substances, and was separated from many of the unwanted products in the culture by simply filtering off the charcoal-streptomycin combination. The conditions were adjusted so that streptomycin would be almost completely adsorbed in a single "batch adsorption" step. A concentrated but still rather impure preparation was obtained when the drug was dissolved from the charcoal with a solvent. If only this batch method were employed, the operations of adsorption, filtration and elution (dissolving off) would have to be repeated many times, employing fresh adsorbent and fresh solvent each time, to obtain material of a high degree of purity.

Tswett's Column

The great value of Tswett's chromatogram is that it provides a very simple device for carrying out repeated adsorptions and elutions continuously. The chromatogram consists essentially of a

glass tube filled with a specially prepared adsorbent. A solution of the material to be fractionated is added to the top of the column of adsorbent. After it has drained into the adsorbent, fresh solvent is percolated through the column. The granules of each layer of adsorbent, and the small volume of liquid that surrounds them, act as a single batch adsorption stage. As the solvent flows away from the first layer of granules and surrounds the granules of the next layer, a second batch adsorption occurs, and so on down the column to give the equivalent of hundreds or thousands of individual stages. In this manner a liquid flowing slowly through a packed column performs continuously a number of operations which would require an impossible amount of time and labor to perform individually.

One important difference should be noted between the single batch adsorption in the process we have described for the purification of streptomycin and a single adsorption stage in the chromatogram. Under these particular conditions the adsorption of streptomycin from the mold culture is essentially a one-way process. In order to dissolve the drug from the adsorbent, a solvent (acidic alcohol) that is different from the original medium is required. In the chromatogram, on the other hand, adsorption and elution frequently take place with the same solvent. Thus the molecules of a given compound in the solution are continuously shuttling back and forth in a reversible equilibrium between the flowing solvent and the stationary adsorbent. When the advancing front of solvent carries these molecules down to an unoccupied layer of adsorbent, the molecules leave the solution and are bound at the surface of the adsorbent. As soon as the influx of new solvent from above dilutes the dissolved material to less than a certain concentration, the adsorbed molecules are again released into solution. Thus a given compound travels down the chromatograph column as a zone, the leading edge of which is continuously inching



CHROMATOGRAPHIC SEPARATION is illustrated by schematic diagrams. In the first diagram a solution containing a mixture of three substances has been placed on top of an adsorbent packed into a glass tube. In the

second the mixture has drained into the adsorbent and fresh solvent has been added. In the third, fourth and fifth the three substances have separated into zones as the solvent has percolated through the column.

ahead while the trailing edge is being gathered up.

The rate of travel of this zone is governed by the relative affinity of a given compound for the solvent and for the adsorbent. If the molecules in question have a great tendency to stick to the adsorbent, the zone will move slowly. If they greatly favor the flowing solvent, on the other hand, a rapidly moving zone will result. The degree of preference of a particular substance for either the solvent or the adsorbent can be measured quantitatively and given a numerical value known as the distribution coefficient. If the molecules of a substance tend to be distributed equally between the solvent and the adsorbent, the distribution coefficient is 1, and a moderately fast-moving zone results. If the coefficient is smaller than 1, the zone moves more slowly; if larger, it moves more rapidly. The actual value of the distribution coefficient depends upon the nature of the adsorbent and of the solvent as well as upon the chemical structure of the compound itself. For a given combination of adsorbent and solvent, however, the coefficient will vary only with the structure of the substance.

Sensitive Separations

It has been found that the magnitude

of the coefficient is frequently very sensitive to slight differences in chemical structure, and it is for this reason that adsorption has been found so useful as a means for separating compounds. In the operation of the Tswett column very small differences in distribution coefficient are translated into appreciable differences in the rate of travel down the column. Thus various substances in a solution, even when very similar to one another chemically, may move down the column at different rates and form separate zones. Within recent years it has been shown that the behavior of zones on a chromatogram can be expressed in mathematical terms, and doubtless this is the "law" whose existence Tswett guessed and mentioned in his first paper.

After the pigments or other substances in a mixture have been separated into zones on the chromatogram, it is necessary to recover the material in each zone for chemical identification. Often this is done simply by pushing the column of adsorbent out of the chromatograph tube with a plunger. As each colored zone of adsorbent emerges from the glass tube, it is cut off with a knife. The segment cut off is then treated with a solvent that removes the desired pigment from the adsorbent, and this solution in turn is evaporated to yield the purified material. There are few more dramatic events

in a laboratory than the crystallization of a much sought compound after this simple procedure.

In recent years the Swiss chemist Tadeus Reichstein, and others, have preferred to pass solvent through the column until the various zones emerge successively from the bottom in solution, instead of pushing the entire column out of the chromatographic tube. Each zone is collected as a separate fraction.

When pigments are being separated, forming zones of different colors, the process can easily be followed visually, as Tswett did in his pioneer experiments. Fortunately, as Tswett predicted, chromatography is not restricted to pigments. The technique works equally well for colorless substances, although some physical or chemical method must be employed to make them distinguishable. For example, some types of compounds show characteristic fluorescent colors when viewed under ultraviolet light. In other cases the fractions from the column, or the extruded column itself, may be treated with various chemical reagents that react with the material in the different zones to produce colors. A radioactive material can be located, after separation, with a Geiger counter; a biologically active fraction can be identified by bioassay, and so on. Actually there is a vast number of ways in which

the progress of chromatographic fractionations can be followed, and new ones are continually being devised.

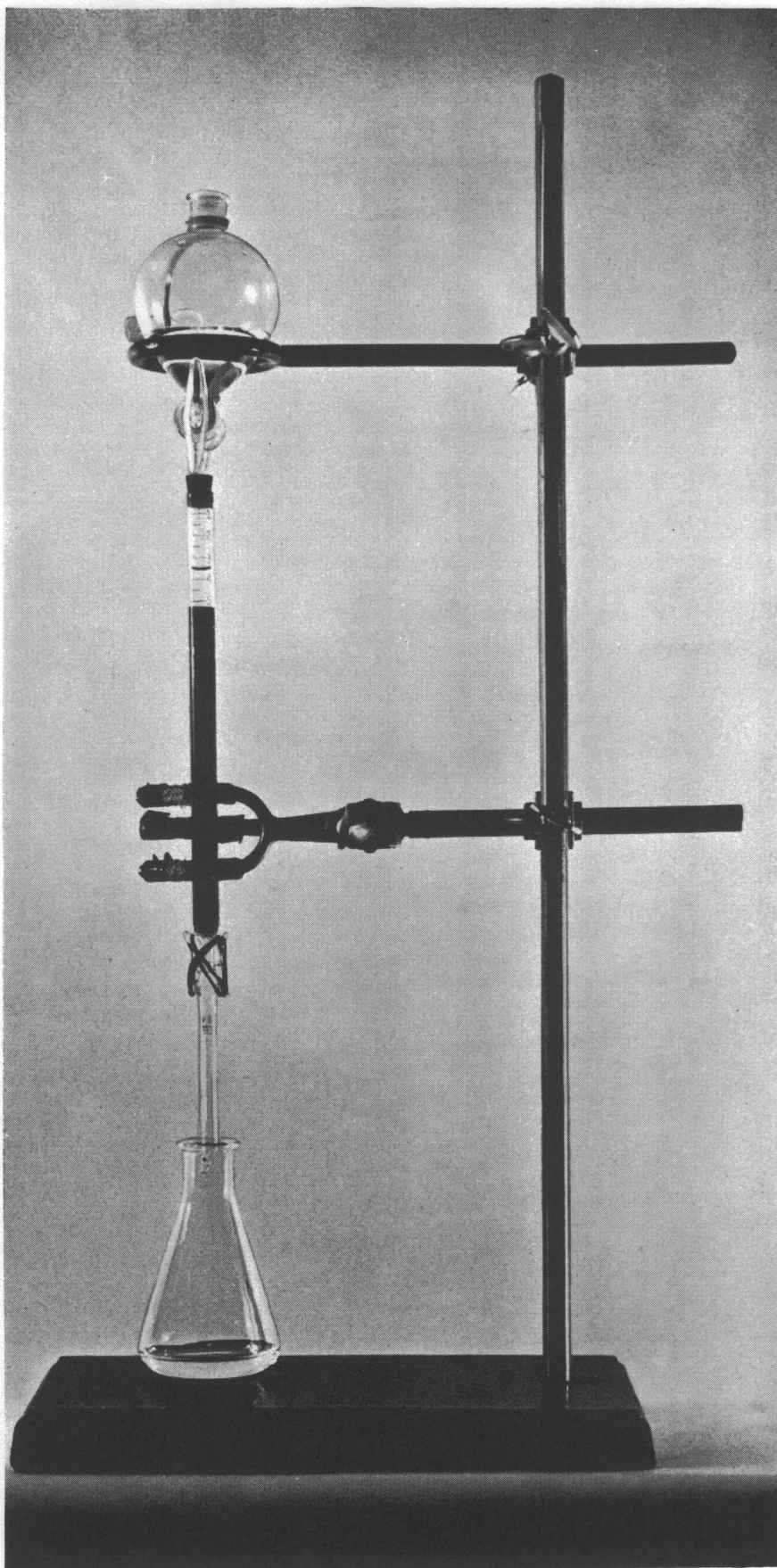
One of the advantages of chromatography is that it requires no elaborate or expensive equipment. All the essential items are readily available in any chemical laboratory. Much of the early and extremely useful work in chromatography was done simply with a long glass tube narrowed at one end and with a few flasks and beakers. Recently additional equipment and procedures have been devised that permit greater efficiency and ease of operation, but few of the modern innovations have modified in any fundamental manner the elegant simplicity of Tswett's original column.

Permutations and Computations

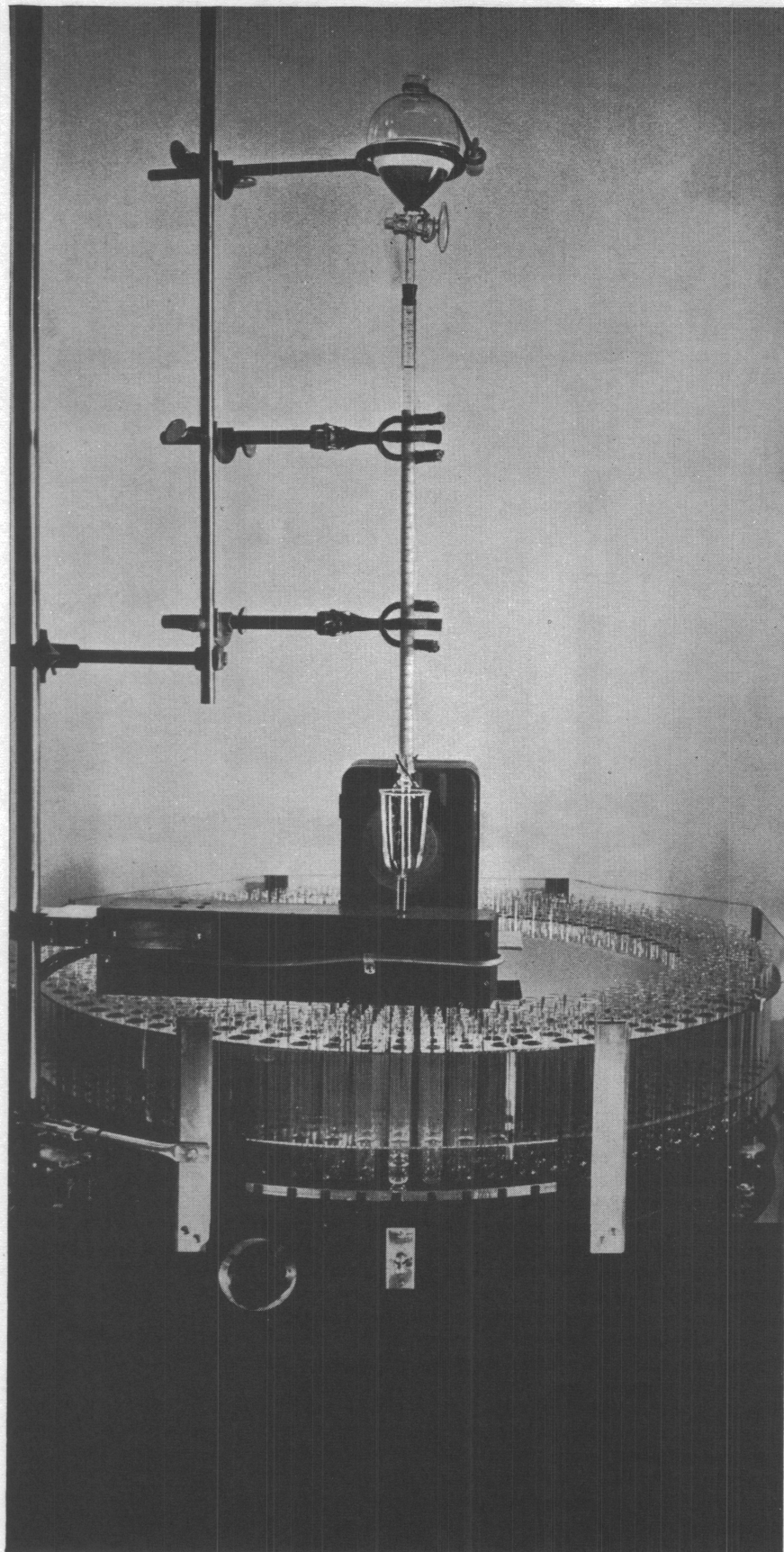
Chromatography is a versatile method. It can be used to fractionate mixtures of gases, liquids or dissolved solids. The method is also a mild one. Although occasionally compounds are altered chemically during the procedure, chromatography has been employed successfully for the separation of some of the most fragile and elusive of substances. It appears increasingly likely that it will eventually be possible to handle chromatographically any group of compounds that can be brought into solution. All that is required is the right combination of solvent and adsorbent.

But that is the difficulty: one of the prime shortcomings of chromatography is that it is not yet possible to predict with assurance what combination of solvent and adsorbent will prove suitable in any given case. The number of possible combinations of solvents and adsorbents is staggering. A partial list of the substances used as adsorbents includes the carbonates of calcium, magnesium and sodium; various forms of charcoal activated in special ways; fuller's earth, bentonite, Lloyd's reagent, talc and innumerable other clays and diatomaceous earths; alumina; silica gel, and a whole host of organic substances such as cellulose, starch, sucrose, inulin, benzoic acid and, more recently, ion-exchange resins. The solvents, too, form an imposing list: water; aqueous solutions of various acids, alkalies and salts; methyl, ethyl, benzyl, propyl, butyl and amyl alcohols; ketonic solvents such as acetone, methyl ethyl ketone and cyclohexanone; hydrocarbons such as benzene, toluene and hexane; the chlorinated hydrocarbons chloroform and carbon tetrachloride; various ethers; the newer derivatives of ethylene glycol, and countless more, alone or in combination.

It is not surprising that for a long time the choice of a chromatographic system was based largely on trial and error. Experience and a few general rules, such



CHROMATOGRAPH TUBE is a simple piece of apparatus that is made in a variety of sizes for different purposes. This small tube is filled with an ion-exchange resin as an adsorbent. At the top of the column is a reservoir of solvent. A small amount of the solvent may be seen on top of the adsorbent.



AUTOMATIC MACHINE is used to collect fractions containing the constituents of a complex mixture. The chromatograph column is in the center; solvent drips from it into collecting tubes on the turntable at the bottom. In the arm beneath the column is a photoelectric cell; when a given number of drops pass it, the turntable rotates and brings an empty tube into position.

as that like tends to adsorb like, have gradually provided some guidance in choosing adsorbent-solvent combinations, and progress is continually being made toward a less empirical procedure. The necessity for much tedious experimentation still is a basic difficulty, and is probably responsible for the fact that the development of the chromatographic method did not proceed more rapidly after Tswett's discovery in 1906.

Milestones

From 1906 until 1931 the Tswett column was used in only a few sporadic investigations. Then R. Kuhn and E. Lederer, two chemists working in Germany, found out with the help of chromatography that crystalline carotene, the yellow pigment of carrots, which for a century had been thought to be a pure substance, was in reality a mixture of two compounds. This important discovery focused attention upon chromatography as a laboratory tool.

Carotene is a precursor of vitamin A, and at that time chemists the world over were busily engaged in attempts to isolate and determine the structure of this and many other vitamins. Soon many investigators in the field of the carotenoids began to use the Tswett column. With its aid Paul Karrer and his associates in Switzerland made important progress in studying the chemistry of vitamin A, and Laszlo Zechmeister of Hungary (now at California Institute of Technology), working with L. Cholnoky, made significant contributions both to carotenoid chemistry and to chromatography. Strain at Stanford, whose comments were quoted at the beginning of this article, did pioneer work in the U. S. in the same fields.

Meanwhile the new tool was also producing major advances in the field of sterol chemistry. The sterols are a large group of substances that play key roles in the life processes of animals and plants. Within this group are found cholesterol, the bile acids, the male and female sex hormones, the plant sterols, vitamin D and the hormones of the adrenal cortex, including desoxycorticosterone and the now much sought cortisone. The sterols have a complicated structure that is capable of innumerable variations. To isolate them in pure form from the intricate mixtures in which they occur in the body, and to elucidate their chemical structure, presented a tremendous challenge to chemists. The eventual successful solution of the problem constitutes one of the truly brilliant chapters in modern chemistry. And in much of this work, destined to be of crucial importance to biochemistry, biology and medicine, the Tswett column was an indispensable aid.

These applications of chromatography all involved the fractionation of mix-

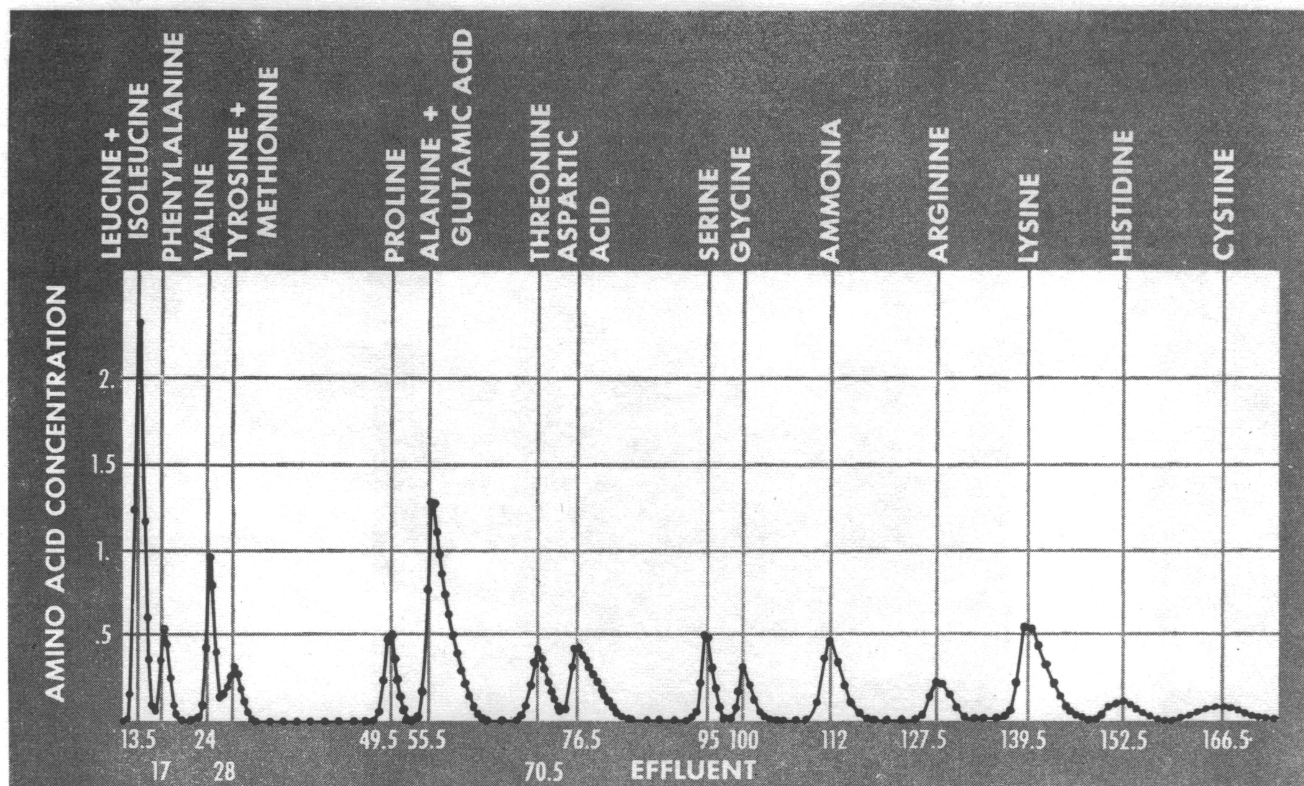


DIAGRAM shows the results of a fractionation by the machine on the opposite page. The mixture was composed of amino acids resulting from the breakdown of the protein bovine serum albumin. Each dot on the curve represents the concentration of one or more amino

acids found in one collecting tube. The identity of the amino acids separated is indicated at the top of the page; their relative quantity is shown by the area under the peaks. The concentration of the amino acids is given in millimoles; the volume of effluent, in cubic centimeters.

tures of substances soluble in fat solvents. The widespread use of chromatography for the separation of compounds soluble in water constitutes another chapter in the history of the method. This chapter was opened about 10 years ago by two young British chemists, A. J. P. Martin and R. L. M. Synge, who devised a technique they have called "partition chromatography."

In partition chromatography the column is packed with a porous solid, such as silica gel, cellulose or starch, which is capable of holding water; the water is usually added to the solid before the chromatograph tube is packed. The substances to be separated are put through the column dissolved in a solvent, such as butyl alcohol, that does not mix with water. In such columns compounds separate into zones just as they do in the Tswett column. As Martin and Synge explained their principle, the compounds were separated by virtue of their distribution between the water, held within the pores of the packing as in a sponge, and the organic solvent flowing through the column. In other words, the water took the place of the adsorbent in the classical Tswett column. Hence the new process was called "liquid-liquid partition" chromatography, to distinguish it from the solid-liquid distribution operating in the Tswett column. Actually it now seems likely that

the two processes do not differ as much as was first thought.

The application of this technique made it possible to separate a number of water-soluble compounds of particular interest to the biochemist, notably the carbohydrates and the amino acids. The new approach also led to improvements in methods for the separation of fat-soluble substances.

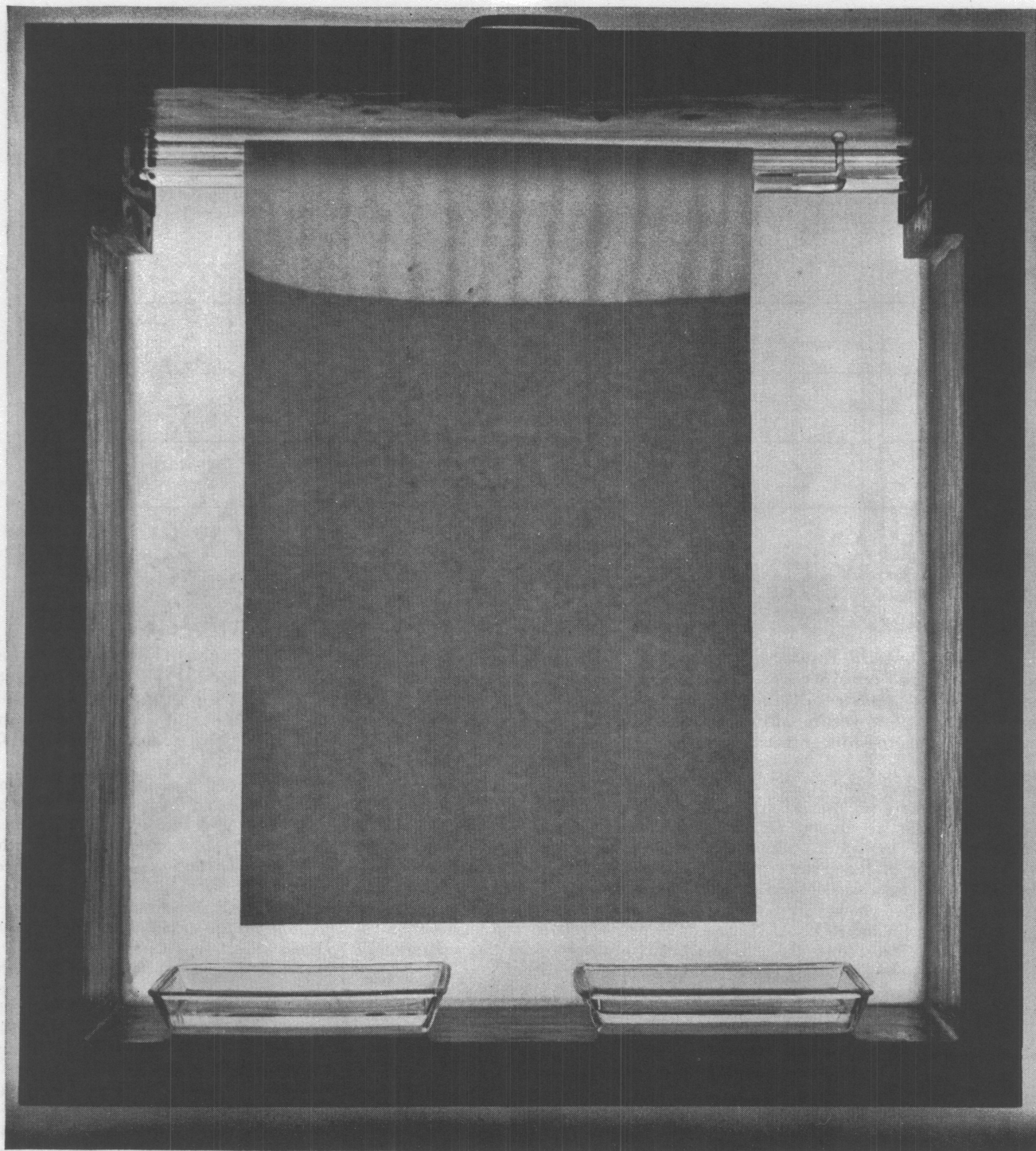
Paper Chromatography

Perhaps the most important development that has arisen from the concept of partition chromatography is the novel and simple technique of paper chromatography. In this method, developed by Martin and his colleagues R. Consden and A. H. Gordon, the separations are carried out on strips or sheets of paper instead of on an adsorbent in a glass tube. Chromatography on paper had been tried before, but the British investigators made it work so effectively that within a few years it has become by far the most popular of all chromatographic methods, and is now being used in chemical laboratories the world over.

An entire paper chromatogram can be carried out on a sheet of paper somewhat larger than a page of this magazine. This corresponds to the chromatographic column. A single drop of the solution to

be fractionated is deposited on a corner of the sheet, and the sheet is then hung, with the drop at the top, in a closed box containing an atmosphere saturated with water vapor. The top edge of the paper is bent over to dip into a trough containing an organic solvent mixture (*see photograph on the next page*). The solvent creeps down the paper by capillarity. After the solvent has traveled the length of the paper, the compounds that were mixed together in the original drop of solution are separated and distributed as a series of spots in a vertical line along the edge of the paper. If, as is frequently the case, the spots still contain a mixture of compounds, making additional purification desirable, the paper may be turned around so that this edge is at the top and a new solvent is then allowed to flow down the sheet. Thus each spot serves as the starting point for a new chromatogram and may yield a series of spots across the sheet. The end result is a so-called "two dimensional" chromatogram, in which compounds are distributed more or less over the whole sheet. This procedure substantially increases the resolving power of the method.

A complex mixture of amino acids, for example, can be separated into spots which give the pattern shown in the background of the illustration on the cover of this issue. Here the spots have been treated with a reagent that reacts



PAPER CHROMATOGRAPHY uses paper in place of the chromatograph column. Here the paper is hung from a trough containing a solvent. As the solvent travels down the paper (*translucent area at top*), the constitu-

ents of a mixture placed near the top will be carried various distances. The paper is suspended in a glass-enclosed box. Within the box a saturated atmosphere is maintained by water and solvent in the dishes at the bottom.

with amino acids to produce a reddish blue color. This is the usual procedure when colorless substances are chromatographed; the paper is sprayed at the end of the experiment with a specific reagent that reacts with the particular class of compounds to yield colored products. Some types of compounds can be seen by examination of the paper

under ultraviolet light. Sometimes the spots may be cut out and the separated compounds washed off the bits of paper for further tests.

How Chemists Use It

The great advantages of paper chromatography are its simplicity, rapidity

and high resolving power. It is being applied to the study of the constituents of human blood and urine in health and disease, to the separation of antibiotics, to the discovery of new amino acids in bacteria and to a great variety of other chemical problems.

Recently paper chromatography has been employed by Melvin Calvin and

Andrew Benson at the University of California in very beautiful investigations of the mechanism of the fixation of carbon dioxide during photosynthesis. They exposed green algae to radioactive carbon dioxide and later chromatographed on paper the mixture of photosynthetic products extracted from the plant cells. The paper chromatogram was then covered with a sheet of X-ray film and left in a dark room for several days. Any products of photosynthesis that had taken up radioactive carbon dioxide would of course show their radioactivity by making spots on the film, which could be seen when the film was developed. From the positions of the spots on the film laid over the paper, the nature of the products that had taken up carbon dioxide could be inferred.

The ultramicro scale upon which paper chromatography can be performed is one of the factors contributing to its usefulness. With the expenditure of only a fraction of a milligram of material, an investigator can frequently obtain information of incalculable value. The small amounts involved mean that it is not possible to isolate a substance in sufficient quantity for identification by conventional chemical procedures. In paper chromatography, identification rests primarily upon the position of the compound upon the paper sheet. Control experiments are required to show that the position of an unknown substance matches exactly that of a known. For this reason paper chromatography cannot provide identification of a completely new compound never before handled by the investigator. Such a substance may appear as a spot in a position not normally occupied by any known compound. When that occurs, the chromatographer must resort to other methods, frequently column chromatography, to isolate and identify the substance. Paper chromatograms do not always provide quantitative results. Frequently they reveal the nature, but not the exact amounts, of the substances present in the mixture chromatographed.

Separation of Amino Acids

Within the past five years certain refinements in the techniques for column chromatography have been developed. These were stimulated by the long-standing need for quantitative work in the field of protein chemistry. They were touched upon in Joseph S. Fruton's article on the proteins in a recent issue of this magazine (*SCIENTIFIC AMERICAN*, June, 1950).

As Fruton pointed out, it is of great importance in protein chemistry to find out precisely the quantities of each of the 20-odd amino acids yielded by the

breakdown of a protein. In the laboratory of the late Max Bergmann at the Rockefeller Institute for Medical Research the authors of the present article had been concerned with this problem for some time. In 1945 they undertook quantitative amino acid analysis with the aid of chromatography. The procedure finally evolved employed columns packed with potato starch and solvents composed of mixtures of certain alcohols with water or aqueous hydrochloric acid. After the amino acid mixture to be analyzed had been added to the prepared chromatograph column, the solvent was run through very slowly until the amino acids had progressed the length of the column and emerged in the effluent issuing from the bottom of the column. The effluent was collected in a large number of successive small fractions of accurately known volume (.5 cubic centimeters per fraction). A quantitative analysis of each fraction was made to determine its amino acid concentration. From these analyses a curve was drawn, showing the concentrations of the various amino acids in the successive fractions (*see diagram on page 7*).

The amino acids, which emerge serially from the bottom of the column, appear on the curve as peaks more or less separated from one another by valleys. Complete separation of all the amino acids in the original mixture is not obtained in a single chromatogram; several emerge together in one peak. Those that overlap can be separated, however, by running additional chromatograms with different solvents. Moreover, the quantity of amino acid present is given accurately by the area under each of the peaks. It has been possible, therefore, to separate and to determine quantitatively each of the 20 or more amino acids found among the cleavage products of a protein. Starch chromatography has proved useful in several laboratories for determining accurately the composition of blood proteins such as hemoglobin and of important hormones of the pituitary gland. It is being employed also in investigations of the metabolism of amino acids using isotopic tracers.

An efficient chromatogram often requires a very slow rate of solvent flow through the column. It takes seven days, for example, to complete an experiment of the type we have been discussing. Obviously it would be too tedious to stand by all this time and collect each fraction by hand. A simple automatic collecting machine has therefore been built. It consists essentially of a circular rack holding the receiving tubes and a photoelectric counter to count the drops emerging from the chromatogram (*see photograph on page 6*). After a set number of drops have emerged, the

turntable moves one notch to bring a new tube under the column. The machine is capable of operating 24 hours a day, thus making practical the performance of experiments of long duration.

Recent Refinements

This article would not be complete without calling attention to other recent developments which appear likely to broaden even further the scope and utility of the chromatographic method. Arne Tiselius and S. Claesson in Sweden have described two new chromatographic techniques known as "frontal analysis" and "displacement development." These procedures, based on new principles, give curves consisting of a series of steps, instead of peaks and valleys. The Swedish workers have devised recording instruments for measuring the stepwise changes in concentration of the effluent. The development of these methods has provided the chemist with additional valuable tools to aid him in his never-ending quest for better ways of resolving complex mixtures. They have already been used to fractionate such diverse mixtures as gaseous hydrocarbons, amino acids, peptides, fatty acids and sugars.

The recent development of organic ion-exchange resins also promises to have profound effects upon the chromatographic method. These ion exchangers were originally prepared to remove dissolved salts from water ("Ion Exchange," by Harold F. Walton; *SCIENTIFIC AMERICAN*, November, 1950). It soon became apparent, however, that they are capable of much wider application. During the war, workers at Oak Ridge and associated university laboratories employed columns packed with ion-exchange resins to separate many of the rare-earth elements formed as fission products in nuclear reactors. Columns of ion-exchange resins are now being used in many laboratories to separate organic compounds. Indeed, current investigations by S. M. Partridge in England and by the authors at the Rockefeller Institute suggest that ion-exchange resins may prove much superior to starch or cellulose as adsorbents for the separation of amino acids and peptides.

It has been said by many, notably the eminent 19th-century physiologist Claude Bernard, that progress in science frequently depends upon the development of good methods. Chromatography furnishes a vivid example of the truth of this statement. It is doubtful that even the fertile imagination of Michael Tswett could have foreseen the great advances in scientific knowledge that have already come, and the greater ones that will surely come in the future, from the application of his chromatograms.

The Authors

WILLIAM H. STEIN and STANFORD MOORE are both professors of biochemistry on the faculty of the Rockefeller Institute, where they began to collaborate on the chemistry of proteins more than two decades ago. Stein, who took his B.S. at Harvard University in 1933, did graduate work in biochemistry under the direction of Edgar G. Miller and Hans T. Clarke at Columbia University, receiving his Ph.D. in 1937. He went to the Rockefeller Institute in 1939 to work in the laboratory of the noted biochemist Max Bergmann. Moore joined Bergmann's laboratory that year, having

acquired a B.A. at Vanderbilt University in 1935 and a Ph.D. in organic chemistry at the University of Wisconsin in 1938. During World War II Stein remained at the Institute to work on problems connected with chemical warfare, while Moore served with the Office of Scientific Research and Development in Washington. They resumed their joint research in 1945.

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